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Botryosphaeria qinlingensis sp. nov. causing oak frogeye leaf spot in China

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Abstract — During our investigation on plant diseases in Qinling Mountain in China, an oak disease with frogeye leaf spot symptoms was discovered. The associated pathogen was characterised by morphological and molecular approaches and is described here as a new species, *Botryosphaeria qinlingensis*.

Key words — *Botryosphaeriaceae, Botryosphaeriales,* taxonomy

Introduction

Botryosphaeria was firstly proposed by Cesati & De Notaris (1863) without designating a type species. Subsequently, *B. berengeriana* (Höhnel 1909) and *B. quercuum* (Theissen & Sydow 1915) were designated as the type, but neither species was included in the original description of the genus. Barr (1972) proposed *B. dothidea* (Moug. ex Fr.) Ces. & De Not. as the type species, because it was one of the original species and an earlier synonym of *B. berengeriana*. *Botryosphaeria dothidea* is now universally accepted as the type of the genus. Slippers & al. (2004) provided a revised description of *B. dothidea* based on the type specimen and fresh collections and designated a neotype and epitype.

Botryosphaeria was restricted to *B. dothidea* and *B. corticis* (Crous & al. 2006), but subsequently, *B. agaves, B. auasmontanum, B. fabicerciana, B. fusispora, B. kuwatsukai, B. minutispermatia, B. pseudoramosa, B. qingyuanensis,*

B. quercus, B. ramosa, B. rosaceae, B. scharifii, B. sinensis, and *B. wangensis* have been accepted in this genus (Abdollahzadeh & al. 2013; Ariyawansa & al. 2016; Chen & al. 2011; Li & al. 2018; Liu & al. 2012; Pavlic & al. 2008; Slippers & al. 2014; Wijayawardene & al. 2016; Xu & al. 2015; Zhou & al. 2016, 2017).

Quercus is an important tree genus that contains many commercial taxa widespread from temperate to subtropical broad-leaved forests in China, such as *Q. acutissima, Q. aliena, Q. aliena* var. *acutiserrata, Q. variabilis,* and *Q. wutaishanica*. A disease with frogeye leaf spot symptoms was discovered on the leaf surface of *Q. aliena* var. *acutiserrata* and *Q. acutissima* (Plate 1). Similar symptoms have been recorded on *Q. dentata, Q. acutissima, Q. aliena, Q. aliena* var. *acutiserrata,* and *Q. variabilis,* and the agents causing those symptoms were identified as *Macrophoma* species (Tian & al. 1991). Here we present an accurate identification based on combined morphology and DNA sequence data, and describe the pathogen as a new species, *Botryosphaeria qinlingensis*.

Materials & methods

Samples and isolates

In this study, diseased oak leaves were collected from *Quercus aliena* var. *acutiserrata* and *Q. acutissima* in Shaanxi Province in China. Single conidial isolates were obtained from conidiomata by removing a mucoid conidial mass from pycnidial ostioles following Jiang & al. (2018). Specimens of the new species were deposited in the Museum of Beijing Forestry University, Beijing, China (BJFC). Axenic cultures were maintained in the China Forestry Culture Collection Center, Beijing, China (CFCC).

Morphological studies

Species identification was based on conidial morphology and fruiting bodies were observed according to Slippers & al. (2013). Fifty spores were selected randomly for measurement using a Leica compound microscope (LM, DM 2500). Morphological or cultural characteristics were observed on PDA after incubation at 25 °C and recorded at 5 and 10 days, including colony color and texture.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from fungal mycelium growing on MEA using a modified CTAB method (Doyle & Doyle 1990). The internal transcribed spacer of rDNA (ITS) was amplified using primers ITS4 and ITS5 (White & al. 1990). The translation elongation factor-1 alpha (tef1-α) was amplified using primers EF1-688F and EF1-1251R (Alves & al. 2008). The β-tubulin gene (tub) was amplified using primers Bt2a and Bt2b (Glass & Donaldson 1995). These regions were amplified and sequenced following Zhang & al. (2009). The PCR products were estimated visually by

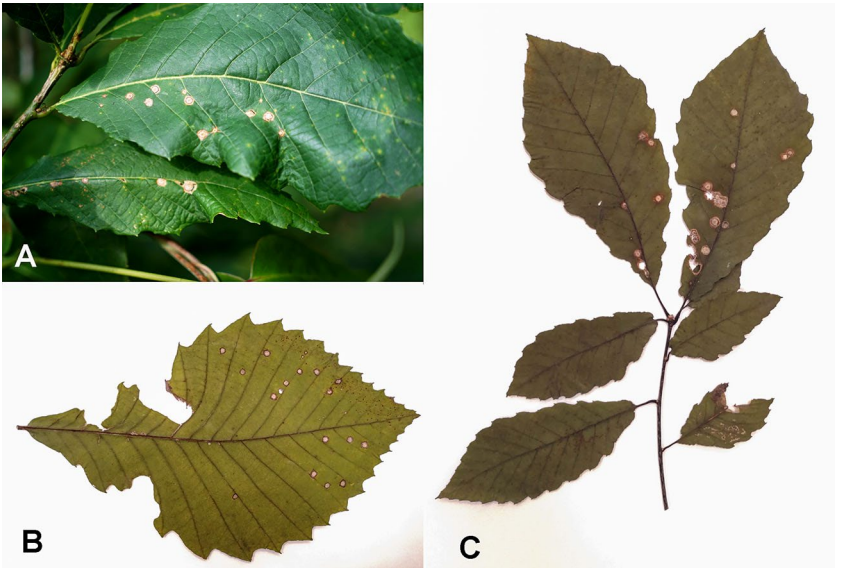

Plate 1. *Botryosphaeria qinlingensis*:
Frogeye disease symptoms on leaves of *Quercus aliena* var. *acutiserrata*.

electrophoresis in 2% agarose gel set to 60 V for 90 min. DNA was sequenced using an ABI PRISM® 3730XL DNA Analyzer with BigDye® Terminater Kit v.3.1 at the Shanghai Invitrogen Biological Technology Company Limited.

DNA sequence analysis

The new sequences generated in this study and the reference sequences of *Botryosphaeria* isolates included in the phylogenetic analyses are summarized in Table 1. *Neofusicoccum luteum* (CBS 110299) was used as outgroup (Li & al. 2018). These sequences were aligned with MAFFT v. 7 (Katoh & Standley 2013) and manually adjusted. Phylogenetic analyses were generated from the combined ITS–tef1α–tub sequences using PAUP v. 4.0b10 (Swofford 2003) for maximum parsimony (MP), PhyML v. 3.0 (Guindon & al. 2010) for maximum likelihood (ML), and MrBayes v.3.1.2 for Bayesian Inference (BI) (Ronquist & Huelsenbeck 2003) following Zhou & al. (2017) and Li & al. (2018).

Results

Molecular phylogeny

The combined ITS, tef1-α, and tub alignment comprised 32 sequences (including one outgroup) and 1344 characters including alignment gaps, of which 1127 were parsimony informative, 95 were variable and parsimony-

Table 1. Isolates used in the molecular analyses.

Species	Isolate number	Host/substrate	Country	GenBank accession numbers		
				ITS	tef1-α	tub
Botryosphaeria agaves	CBS 133992	*Agave* sp.	Thailand	JX646791	JX646856	JX646841
	MFLUCC 10-0051	*Agave* sp.	Thailand	JX646790	JX646855	JX646840
B. auasmontanum	CBS 121769	*Acacia mellifera*	Namibia	EU101303	EU101348	—
B. corticis	CBS 119047	*Vaccinium corymbosum*	USA	DQ299245	EU017539	EU673107
	ATCC 22927	*Vaccinium* sp.	USA	DQ299247	EU673291	EU673108
B. dothidea	CBS 115476	*Prunus* sp.	Switzerland	AY236949	AY236898	AY236927
	CBS 110302	*Vitis vinifera*	Portugal	AY259092	AY573218	EU673106
B. fabicerciana	CBS 127193	*Eucalyptus* sp.	China	HQ332197	HQ332213	KF779068
	CBS 127194	*Eucalyptus* sp.	China	HQ332198	HQ332214	KF779069
B. fusispora	MFLUCC 10-0098	*Entada* sp.	Thailand	JX646789	JX646854	JX646839
B. kuwatsukai	CBS 135219	*Malus domestica*	China	KJ433388	KJ433410	—
	LSP 5	*Pyrus* sp.	China	KJ433395	KJ433417	—
B. minutispermatia	GZCC 16-0013	Dead wood	China	KX447675	KX447678	—
	GZCC 16-0014	Dead wood	China	KX447676	KX447679	—
B. pseudoramosa	CERC1999	*Eucalyptus* hybrid	China	KX277988	KX278093	KX278197

	CGMCC3.18739	*Eucalyptus* hybrid	China	KX277989	KX278094	KX278198
B. qingyuanensis	CGMCC3.18742	*Eucalyptus* hybrid	China	KX278000	KX278105	KX278209
	CGMCC3.18743	*Eucalyptus* hybrid	China	KX278001	KX278106	KX278210
B. qinlingensis	CFCC 52984	*Quercus aliena* var. *acutiserrata*	China	MK434301	MK425020	MK425022
	CFCC 52985	*Quercus acutissima*	China	MK434302	MK425021	MK425023
B. quercus	MFLUCC 14-0459	*Quercus* sp.	Italy	KU848199	—	—
B. ramosa	CBS 122069	*Eucalyptus camaldulensis*	Australia	EU144055	EU144070	KF766132
B. rosaceae	CGMCC3.18007	*Malus* sp.	China	KX197074	KX197094	KX197101
	CGMCC3.18008	*Amygdalus* sp.	China	KX197075	KX197095	KX197102
B. scharifii	CBS 124703	*Mangifera indica*	Iran	JQ772020	JQ772057	—
	CBS 124702	*Mangifera indica*	Iran	JQ772019	JQ772056	—
B. sinensis	CGMCC3.17723	*Morus* sp.	China	KT343256	KU221233	KX197107
	CGMCC3.17724	*Juglans regia*	China	KT343255	KU221234	KX197108
B. wangensis	CGMCC3.18744	*Cedrus deodara*	China	KX278002	KX278107	KX278211
	CGMCC3.18745	*Cedrus deodara*	China	KX278003	KX278108	KX278212
Dichomera saubinetii	CBS 990.70	Dead branch	Netherlands	KF766153	KF766396	—
Neofusicoccum luteum	CBS 110299	*Vitis vinifera*	Portugal	AY259091	AY573217	DQ458848

uninformative, and 122 were constant. The MP analysis produced 50 equally most parsimonious trees, with the first tree (TL = 261, CI = 0.893, RI = 0.905, RC = 0.808) shown in Plate 2. The ML and BI phylogenetic trees were topologically similar to the MP tree. The new species appeared in a single clade with high bootstrap support (Plate 2).

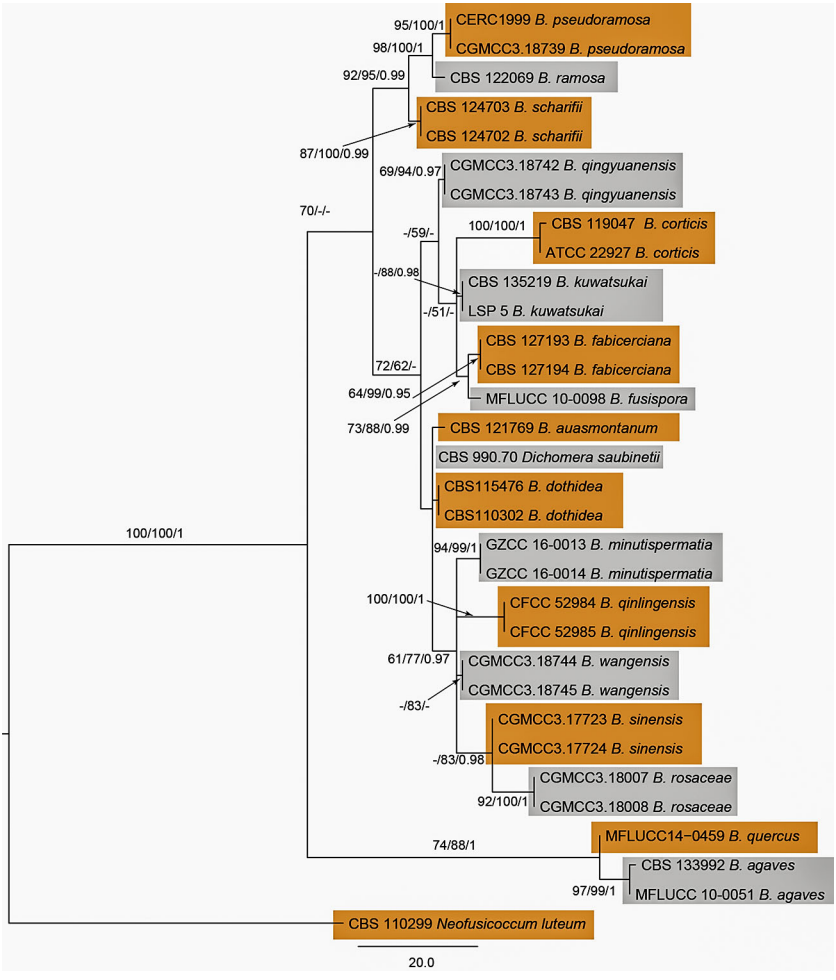

Plate 2. Phylogram of *Botryosphaeria* based on combined ITS, tef1, and tub genes. Bootstrap support values >50% and posterior probability values ≥0.95 are presented as MP/ML/PP, above branches. Scale bar = 20 nucleotide substitutions.

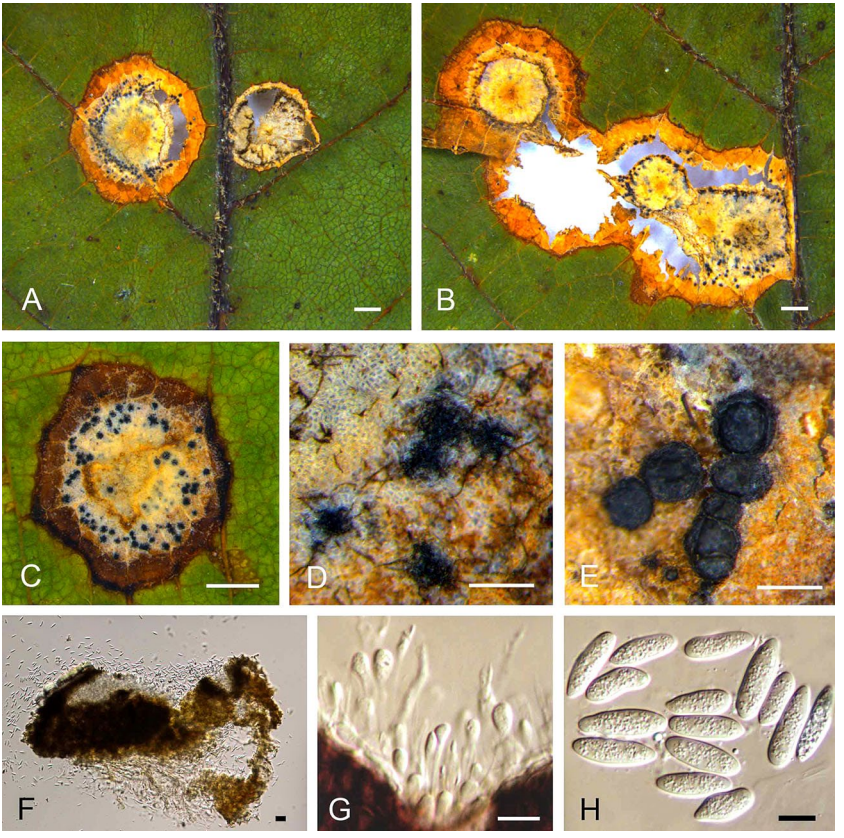

Plate 3. *Botryosphaeria qinlingensis* from *Quercus aliena* var. *acutiserrata* (BJFC-S1576, holotype). A–D. Conidiomata; E. Transverse sections through conidiomata; F. Longitudinal section through a conidioma; G. Conidiogenous cells and developing conidia; H. Conidia. Scale bars: A–C = 1 cm; D, E = 2 mm; F–H = 10 μm.

Taxonomy

Botryosphaeria qinlingensis C.M. Tian & L.Y. Liang, **sp. nov.** Plate 3

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Differs from *Botryosphaeria wangensis* and *B. minutispermatia* by its wider conidia.

Type: China, Shaanxi Prov., Ningshan County, Qinling Mountain, Huoditang forest park, 33°26′07″N 108°26′48″E, 1570 m a.s.l, on leaves of *Quercus aliena* var. *acutiserrata*, 3 July 2018, coll. N. Jiang & C.M. Tian (**Holotype,** BJFC-S1576; ex-type culture CFCC 52984; GenBank MK434301, MK425020, MK425022).

Etymology: *qinlingensis*, named after the Qinling Mountain.

Sexual morph: Undetermined. Asexual morph: Conidiomata formed on oak leaf spot, stromatic, brown to black, 120–250 μm diam, forming a botryose aggregate of up to 15 (sometimes solitary) globose with a central ostiole, semi-immersed to superficial. Paraphyses, hyaline, aseptate, up to 35 μm long, 3–5 μm diam. at the base tapering to acutely rounded apices, 1–2 μm diam. at the tip. Conidiophores absent. Conidiogenous cells holoblastic, discrete, hyaline, cylindrical to lageniform, phialidic with periclinal thickening, 4.5–10.5 × 1.5–3 μm. Conidia hyaline, thin-walled, smooth with granular contents, unicellular, aseptate, ellipsoid to fusoid, base subtruncate to bluntly rounded, (14.3–)19.2–24.8(–30.0) × (6.1–)7.1– 8.4(–9.5) μm, L/W = (2.0–)2.4–3.3(–3.7) (n = 100). Colonies initially white with fluffy, aerial mycelium, becoming black on the surface after 5 days; reverse side of the colonies dark-brown.

Additional specimen examined: **CHINA**, **Shaanxi Prov.**, **Ningshan County**, Qinling Mountain, Shibazhang village, 33°26′10″N 108°26′42″E, 1700 m a.s.l, on leaves of *Quercus acutissima*, 7 July 2018, coll. N. Jiang & C.M. Tian (BJFC-S1577; living culture CFCC 52985; GenBank MK434302, MK425021, MK425023).

Host/Distribution: on leaves of *Quercus aliena* var. *acutiserrata* and *Q. acutissima* in China.

Note: The two isolates of *B. qinlingensis* clustered in a well-supported clade (MP/ML/BI = 100/100/1) in Plate 2 and appeared closely related to *B. wangensis* G.Q. Li & S.F. Chen and *B. minutispermatia* Ariyaw. & al. However, *B. qinlingensis* can be morphologically distinguished from *B. wangensis* and *B. minutispermatia*, which differ by their narrower conidia (*B. wangensis*, 5.5–6.5 μm, Li & al. 2018; *B. minutispermatia*, 3–4 μm, Ariyawansa & al. 2016).

Discussion

In this study, disease samples with typical frogeye leaf spot symptom were observed and collected from *Quercus aliena* var. *acutiserrata* and *Quercus acutissima* in Qinling Mountain in China. Based on DNA sequence data of combined ITS, tef1-α, and tub loci, *Botryosphaeria qinlingensis* is proposed here as the pathogen causing oak frogeye leaf spot disease.

Although the sexual state of *Botryosphaeria qinlingensis* was absent, we can still distinguish this pathogen from other *Botryosphaeria* species by conidial dimension (Table 2), supplemented by host information. Another species, *Botryosphaeria quercus*, was recorded on oak leaves collected from Italy and described with only *Dichomera*-like conidia, but we can separate *B. quercus* and *B. qinlingensis* in the phylogenetic tree (Plate 2).

Table 2. Conidial size of accepted *Botryosphaeria* species.

Species	Conidial size (μm)	L/W	Reference
B. agaves	—	—	Liu & al. 2012
B. auasmontanum	(8.1–)8.8–11.3(–13) × (2.5–)2.9–3.9(–5)	3	Slippers & al. 2014
B. corticis	(20.5–)23.5–32.5(–34.5) × (5.0–)5.5–7(–7.5)	4.5	Phillips & al. 2006
B. dothidea	(20–)23–27(–30) × 4–5(–6)	4.9	Slippers & al. 2004
B. fabicerciana	(16.5–)19.5–24.5(–26) × (4.5–)5–6.5(–7.5)	3.8	Chen & al. 2011
B. fusispora	16–22 × 4–5.5	4	Liu & al. 2012
B. kuwatsukai	(18.5–)20–24.5(–26) × 5–7(–8)	3.6	Xu & al. 2015
B. minutispermatia	8–14 × 3–4	3.7	Ariyawansa & al. 2016
B. pseudoramosa	(8–)10–13(–16) × (4–)4.5–5(–6)	2.5	Li & al. 2018
B. qingyuanensis	(15–)19.5–24.5(–28.5) × (5–)6–6.5(–7.5)	3.5	Li & al. 2018
B. qinlingensis	(14.3–)19.2–24.8(–30) × (6.1–)7.1–8.4(–9.5)	2.4–3.3	This study
B. quercus	10–24 × 6–10	—	Wijayawardene & al. 2016
B. ramosa	(11–)12–15(–16) × (4.7–)5–6(–7)	2.3	Pavlic & al. 2008
B. rosaceae	20–31 × 6–8	3.9	Zhou & al. 2017
B. scharifii	(11.5–)13–17(–19) × 4–6.5	2.7	Abdollahzadeh & al. 2013
B. sinensis	(15–)19–29 × 5–7	4.1	Zhou & al. 2016
B. wangensis	(20.5–)22–26(–29) × (4.5–)5.5–6.5(–7.5)	3.9	Li & al. 2018

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